

Effects of Hot-Water Extract from *Sargassum* sp. on Antibacterial Activity, Non-specific Immunity and TBARs Production on Asian Seabass (*Lates calcarifer*, Bloch)

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ABSTRACT

Hot-water extract of *Sargassum* sp., a marine macro-algae, was examined for its effects on non-specific immune responses and lipid peroxidation (TBARs) production in Asian seabass (*Lates calcarifer*, Bloch) upon bacterial infection. Antibacterial activity of the extract was tested in vitro against four common pathogens of Asian seabass, namely *Streptococcus iniae*, *Vibrio alginolyticus*, *V. parahaemolyticus*, and *V. vulnificus*, using micro-dilution broth technique. Thereafter, the *in vivo* humoral non-specific immune stimulatory effects and TBARs production upon *S. iniae* infection were investigated. Asian seabass with average weight of 167.68 ± 34.75 g were divided into four groups (15 fish per group). Group 1 acted as the negative control, whereas Group 2 was treated with *Sargassum* sp. extract at a dose of 50 mg kg^{-1} of fish, Group 3 was infected with *S. iniae* ($2 \times 10^3 \text{ CFU fish}^{-1}$), and Group 4 received both the extract and *S. iniae*. At 2, 24 and 48 hours after treatment, blood was withdrawn from the caudal vein of 5 fish in each group to examine haematocrit, lysozyme and alternative complement activities, while their livers were rapidly removed to determine TBARs. No antibacterial activity of *Sargassum* sp. extract was observed *in vitro*. However, the *S. iniae* injection caused an increase in serum TBARs ($P < 0.05$) in fish which did not receive the seaweed extract, indicating induced oxidative stress. Serum alternative complement and lysozyme levels in groups receiving the seaweed extract became significantly higher than those of their corresponding controls after 24 and 48 hours post-treatment, respectively. The present study indicates that the hot-water extract from *Sargassum* sp. enhances non-specific immune responses and suppresses lipid peroxidation in Asian seabass.

Keywords: *Sargassum* sp., Asian seabass, antibacterial activity, immune responses

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INTRODUCTION

Bacterial infection is a major problem in fish culture worldwide. Asian seabass (*Lates calcarifer*), one of Thailand's economically cultured fish, is also susceptible to various pathogenic bacteria (Suanyuk *et al.*, 2010). Nowadays, antibiotics have been used to treat infectious bacterial diseases in aquaculture farms. However, use of these chemicals lead to pollution, damage of the ecological system in the surrounding environment of fish farms, and drug resistance in fish against pathogenic agents. The high proportion of antibiotic-resistant bacteria which persist in sediments and farm environments may provide a threat to fish farms because they can serve as sources of antibiotic-resistant genes for fish pathogens in the vicinity of the farms (Furushita *et al.*, 2003). Because resistant bacteria may be transferred to humans and are capable of transferring their resistant elements to opportunistic human pathogens, the implementation of efficient strategies to contain and manage resistant gene emergence and spread is critical. In addition to the potential effects on human health, inefficiency of antibiotic treatment of fish diseases leads to significant economic losses (Agnew and Barnes, 2007). The use of natural compounds to promote non-specific immune response in fish and to prevent the outbreak of disease has been considered as one of the best solutions to develop a sustainable and chemical or antibiotic-free aquaculture system.

Marine algae extracts contain secondary metabolites with anti-tumor, antibacterial and immunostimulatory activities (Nahas *et al.*, 2007). *Sargassum* sp. is a brown seaweed, growing mostly in tropical waters, and is found in both the Gulf of Thailand

and Andaman Sea. It is mainly used as food and fertilizer for palm trees. *Sargassum* sp. contains alginic acid as a major polysaccharide, having high anticoagulant and antimicrobial properties (Patra *et al.*, 2008). A previous study by Yangthong *et al.*, (2009) demonstrated that the hot-water extract of *Sargassum* sp. collected from a coastal area in Gulf of Thailand had the highest antioxidant activity compared to *Caulerparacemosa* var. *macrophysa*, *Gracilaria tenuistipitata* var. *tenuistipitata*, and *Ulva lactuca*.

It has been reported that seaweed extracts could increase the survival of crustaceans against pathogen and enhance their immunity. The administration of hot-water extract of *Sargassum duplicatum* through immersion or injection increased the immune response of *Litopenaeus vannamei* (Pacific white shrimp) by increasing the total haemocyte count, phenoloxidase activity, respiratory burst, and resistance against *Vibrio alginolyticus* (Yeh *et al.*, 2006). In accordance with these, Huynh *et al.* (2011) reported that the administration of *S. hemiphyllum* by immersion increased the resistance of *L. vannamei* against *V. alginolyticus* and white spot syndrome virus (WSSV). The injection of hot water extract from *S. autumnale* at a dose of 50 mg kg⁻¹ fish increases the resistance of carp to *Edwardsiella tarda* infection and enhances the resistance of yellowtail (*Seriola quinqueradiata*) against *Streptococcus* sp. infection (Fujiki *et al.*, 1992). However, there has been no information on the effect of hot-water extract of seaweed on non-specific immune responses in marine fish so far. Thus, this present study was conducted to investigate the effects of hot water extracts from *Sargassum* sp. on non-specific immunity and TBARs production in Asian seabass.

MATERIALS AND METHODS

Preparation of hot-water extract of Sargassum sp.

Samples of *Sargassum* sp. (SG-0044) were collected from the coastal areas of Songkhla Province, south of Thailand. After harvesting, samples were put in plastic bags, placed on ice and transported to the laboratory. Samples were washed thoroughly with fresh water to remove salt, sand, and epiphytes, then air-dried and pulverized. Subsequently, 10 g of the algal powder was added to 100 ml of deionized water and autoclaved for 3 hours (Huang and Lee, 2005). The extract was then filtered through a nylon mesh (300 μ m pore size), spray-dried and kept in desiccator cabinets. The same batch of extract powder was used in all experiments.

Antibacterial assay

Hot-water extract of *Sargassum* sp. was tested for its antibacterial activity against fish pathogenic bacteria of Asian seabass, namely *Streptococcus iniae*, *Vibrio alginolyticus*, *V. parahemolyticus* and *V. vulnificus*, using the micro-dilution broth technique (Hayder *et al.*, 2005). Oxytetracycline was used as a standard antibiotic.

In vivo immune-stimulation study: experimental design

Asian seabass fingerlings from a private farm in Songkhla Province were transported to the rearing facility of the Coastal Aquatic Animal Health Research Institute and acclimated by feeding twice daily with commercial feed. After two weeks, fish with an average weight of 167.68 \pm 34.75 g (mean \pm SD) were divided into four groups (15 fish per group). Group 1 and 3 were intra-peritoneally injected with 100 μ l

of 0.85% NaCl while Groups 2 and 4 were injected with 100 μ l of *Sargassum* sp. extract in 0.85% NaCl at a dose of 50 mg kg $^{-1}$ fish. After 24 hours, Groups 1 and 2 were re-injected as before, while Groups 3 and 4 were injected with 100 μ l each of 0.85% NaCl containing *Streptococcus iniae* (2x10³ CFU/fish). At 2, 24 and 48 hours after the second injection, blood was withdrawn from the caudal veins of 5 fish in each group and their livers were rapidly removed. The blood samples were divided into two portions: the first one was mixed with heparin at a dose of 150 units ml $^{-1}$ for hematocrit determination, while the serum from the remaining portion was stored at -80°C for the alternative complement and lysozyme assays. Livers were excised, rinsed in cold normal saline and stored at -20°C for lipid peroxidation assay.

Alternative complement activity assay

This assay was modified from Yano (1992) and Ortuno *et al.* (1998) by using rabbit red blood cells (RaRBC). Briefly, the RaRBC were washed and adjusted to 2x10⁸ cells ml $^{-1}$ in ethylene glycol tetraacetic acid-magnesium-gelatin veronal buffer (0.01M). Exactly 100 μ l of the RaRBC suspension was lysed with 3.4 ml of distilled water and the absorbance of the haemolysate was measured at 414 nm against distilled water to obtain the 100% lysis value. The test serum was appropriately diluted and different volumes ranging from 40 μ l to 100 μ l were made up to 100 μ l total volume before being allowed to react with 40 μ l of RaRBC in a test tube. The supernatant was collected and measured for its absorbance at 405 nm. A lysis curve was obtained by plotting the percentage of haemolysis against the volume of serum added. The dilution corresponding to 50% haemolysis ml $^{-1}$ was expressed as ACH50 units ml $^{-1}$.

Lysozyme activity assay

Serum lysozyme activity was measured according to the methods of Obach *et al.* (1993) and Demers and Bayne (1997) based on the lysis of lysozyme sensitive *Micrococcus lysodeikticus*. The absorbance was measured every 30 seconds for 5 min at 450 nm. Lysozyme concentrations were calculated from a standard curve of known lysozyme from hen egg white and reported as μg lysozyme ml^{-1} serum.

Lipid peroxidation assay

Lipid peroxidation (TBARs) was measured as described by Jaczynski and Park (2003), in which 0.1 g liver sample was homogenized in 1 ml of ice cold 50 mM potassium phosphate buffer, pH 7.8 and centrifuged at 7,000 \times g for 20 min at 4°C. Then, 0.25 ml of the homogenate was mixed with 1.25 ml of reagent assay mixture (1% butylated hydroxytoluene, 8% sodium dodecyl sulphate, and 0.8% thiobarbituric acid in 20% acetic acid) and put in a boiling waterbath for 30 min. The samples were cooled down to room temperature and centrifuged at 2,000 \times g for 5 min. The supernatants were then measured for absorbance at 532 nm using malondialdehyde (MDA) as a standard. The TBARs was reported as nmol MDA g^{-1} liver.

Statistical Analysis

All data are presented as means \pm standard deviation (SD) and analyzed by one-way ANOVA. Differences in mean values were considered significant at $P < 0.05$.

RESULTS

Antibacterial activity

The results of the antibacterial activity are shown in Table 1. The hot water extract from *Sargassum* sp. was not able to inhibit the growth of bacteria.

Hematocrit

The results of the hematocrit determination are shown in Table 2. The hematocrit of all four groups of Asian seabass at 2, 24 and 48 hours ranged from 28.40 ± 2.87 to $35.00 \pm 1.67\%$ and were not significantly different ($P > 0.05$).

Complement activity

The results of the assay of complement activity are shown in Figure 1. The complement activity in all four groups of fish at 2, 24 and 48 hours ranged from 15.18 to 58.78 unit ml^{-1} of serum. There were some significant differences between the treated and control groups. The seaweed extract stimulated complement activity in both infected and non-infected fish (Groups 2 and 4) at 24 hours post-treatment. Bacterial infection, however, reduced the complement activity in the fish serum as seen in fish from Groups 3 and 4.

Lysozyme activity

The results of the lysozyme activity assay are shown in Figure 2. The lysozyme activity of the Asian seabass in all four groups at 2, 24 and 48 hours ranged from 22.70 to 33.14 μg ml^{-1} serum. The seaweed extract stimulated the same level of activity in both infected and non-infected fish (Groups 2 and 4) at 48 hours post-treatment and the difference was found to be significant ($P < 0.05$), while no significant differences among the treatments were found in the activity at 2 and 24 hours.

Table 1. Antimicrobial activity of hot water extract from *Sargassum* sp. by microdilution broth technique

Fish pathogenic bacteria	Concentration (mg ml ⁻¹)	Antimicrobial activity	
		Oxytetracycline	<i>Sargassum</i> extract
<i>Streptococcus iniae</i>	1	✓	x
	0.5	✓	x
	0.25	✓	x
	0.12	✓	x
	0.06	✓	x
	0.03	✓	x
	0.015	✓	x
	0.007	✓	x
<i>Vibrio alginolyticus</i>	1	✓	x
	0.5	✓	x
	0.25	✓	x
	0.12	✓	x
	0.06	✓	x
	0.03	✓	x
	0.015	✓	x
	0.007	✓	x
<i>Vibrio parahaemolyticus</i>	1	✓	x
	0.5	✓	x
	0.25	✓	x
	0.12	✓	x
	0.06	✓	x
	0.03	✓	x
	0.015	✓	x
	0.007	✓	x
<i>Vibrio vulnificus</i>	1	✓	x
	0.5	✓	x
	0.25	✓	x
	0.12	✓	x
	0.06	✓	x
	0.03	x	x
	0.015	x	x
	0.007	x	x

✓:bacterial growth not visible or able to inhibit the bacteria, x :bacterial growth visible or unable to inhibit the bacteria.

Table 2. Hematocrit value (%) at different times after the second injection

Time after injection (h)	Group			
	1	2	3	4
2	30.80 ± 2.90	30.80 ± 1.60	29.80 ± 2.71	29.80 ± 0.98
24	33.40 ± 2.42	35.00 ± 1.67	33.25 ± 1.48	32.80 ± 1.60
48	31.40 ± 3.26	31.40 ± 2.65	28.40 ± 2.87	28.67 ± 1.25

Values are mean ± SD (n = 5).

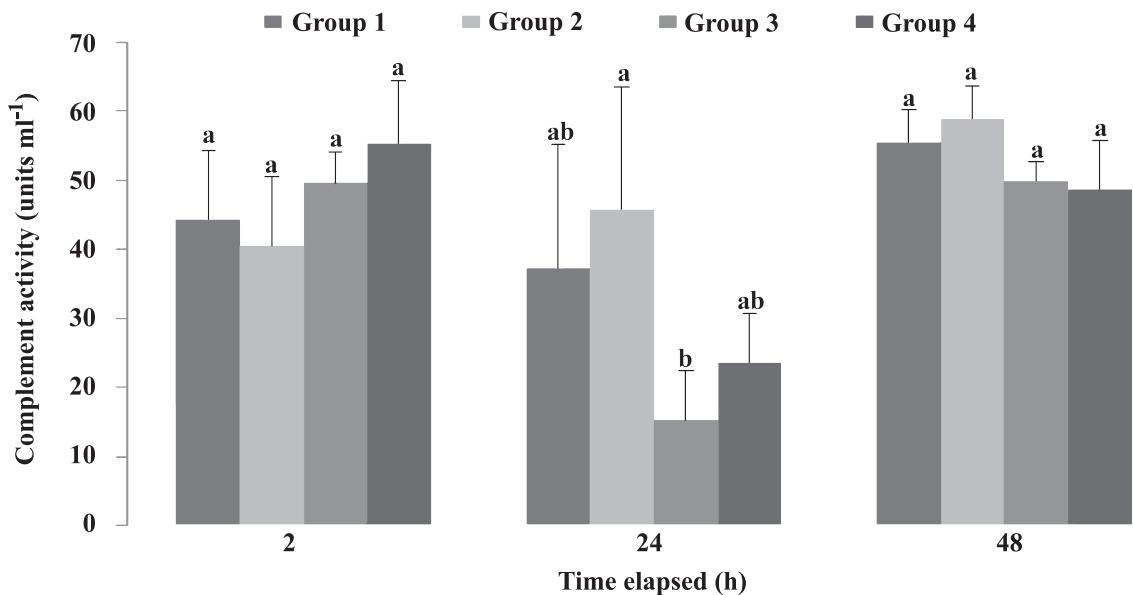


Figure 1. Serum alternative complement activities of the four different groups of experimental fish. Each bar represents mean \pm SD. Data at the same exposure time with different letters are significantly different ($P<0.05$) among groups.

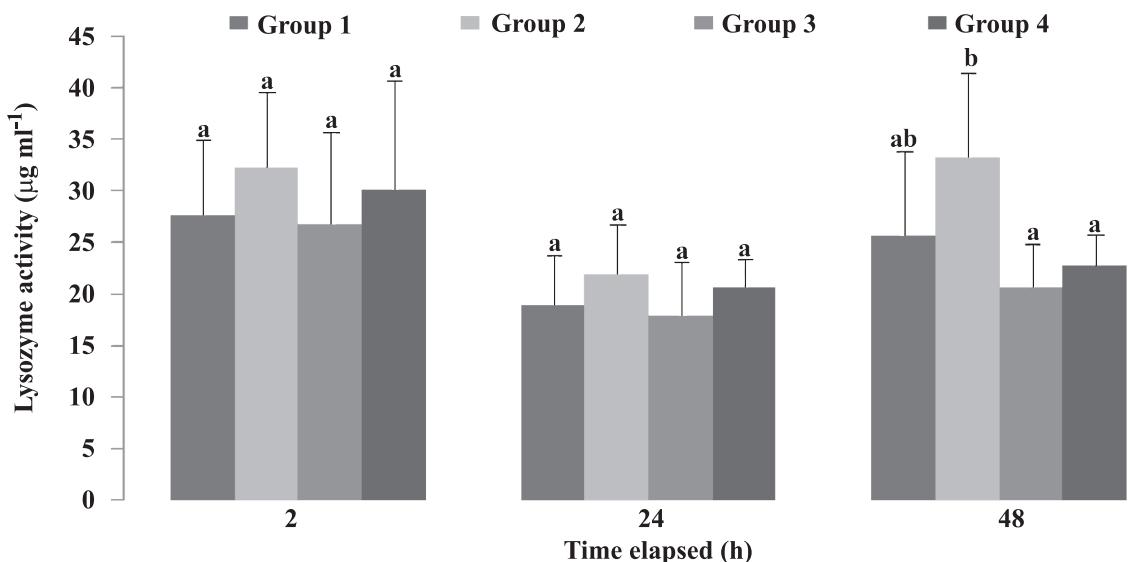


Figure 2. Serum lysozyme activities of the four different groups of experimental fish. Each bar represents mean \pm SD. Data at the same exposure time with different letters are significantly different ($P<0.05$) among groups.

TBARS production

The result of the TBARS assay is shown in Figure 3. The TBARS of the four groups of seabass at 2, 24 and 48 hours ranged from 174.88 to 271.36 nmol MDA g⁻¹ liver. At 2 hours, the TBARS values were

not significantly different while at 24 hours, TBARS of group 3 was significantly higher than that of the other groups. At 48 hours, the TBARS value of the group injected with NaCl only (Group 1) was significantly higher than that of the other groups.

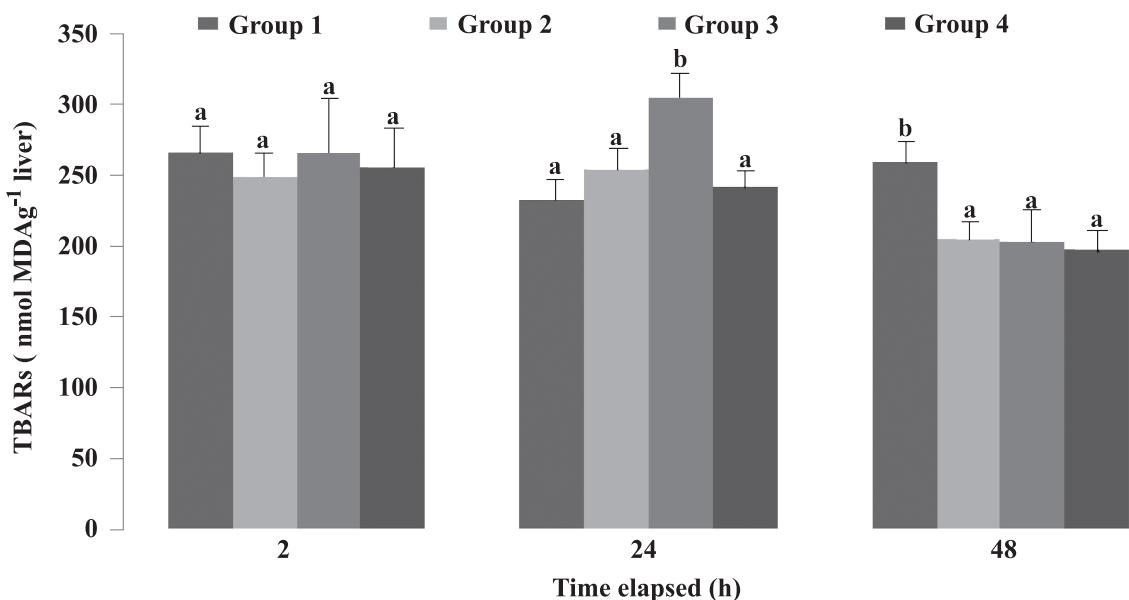


Figure 3. TBARS of the four different groups of experimental fish. Each bar represents mean \pm SD. Data at the same exposure time with different letters are significantly different ($P < 0.05$).

DISCUSSION

The hot-water extract of *Sargassum* sp. in the present study contained mainly “fucose” which is not surprising since the sulfated L-fucose is the main component of the polysaccharide in the cell wall of brown seaweeds (Kantachumpoo and Chirapart, 2010). It has been reported that fucose-rich sulfated polysaccharides from *Sargassum* sp. have beneficial effects such as for anti-inflammation, anticoagulant (Ale *et al.*, 2012) and anti-microorganism (Bertea and Mulloy, 2003). In contrast, Kantachumpoo

and Chirapart (2010) have shown that crude polysaccharides with high sulfate contents did not inhibit microorganisms but promoted their growth since the high carbohydrate content in the extract could be used as a carbon source for microbial growth. Therefore, water-based extracts of seaweed may not be a good choice as an antimicrobial agent.

The results from the present study which showed no antibacterial activity of the hot-water extracts of dried *Sargassum* sp. against both gram positive (*Streptococcus iniae*) and negative (*Vibrio alginolyticus*, *V. parahemolyticus*, *V. vulnificus*) pathogenic

bacteria for Asian seabass are in accordance with those of aqueous extracts of dried seaweeds from Rhodophyta, Phaeophyta and Chlorophyta (Bansemir *et al.*, 2006; Patil *et al.*, 2010; Priyadharshini *et al.*, 2011). However, Lavanya and Veerappan (2011) reported that there was antibacterial activity of fresh algae aqueous extracts (*Codium decorticatum*, *Caulerpa scalpelliformis*, *Gracilaria crassa*, *Acanthophora spicifera*, *Sargassum wightii* and *Turbinaria conoides*) against human pathogenic bacteria. It might be possible that the type of seaweed (fresh or dry) influenced the antibacterial activity of the extract. The antimicrobial activity of seaweeds may be related to several factors such as age, growth stages, habitat, season of algal collection, type of algae (fresh or dry), thallus composition, extraction methods and type of solvent used for extraction (Robles-Centeno *et al.*, 1996; Padmakumar and Ayyakkarmu, 1997; Kumar and Rengasamy, 2000; Asker *et al.*, 2007; Manilal *et al.*, 2009; Wei *et al.*, 2011).

It is clear that using organic solvents will provide a higher efficiency for extracting antimicrobial compounds compared to water-based methods. Zubia *et al.* (2008) reported that the aqueous and ethanolic extracts of fresh seaweeds such as *Sargassum mangarevense* and *Turbinaria ornata* showed antibacterial activity against gram positive *Staphylococcus aureus* while there was no activity against gram negative bacteria. In addition, they noted that the extracts were more effective against gram-positive than gram-negative bacteria, which have a more complex cell wall structure. However, the side effects of using solvent extracted seaweeds on health of animal are unclear. Therefore, water extraction seems to be more interesting.

In the present study, the hematocrit level, lysozyme and complement activities of Asian seabass injected with 50 mg kg⁻¹ *Sargassum* sp. hot water extract increased, while lipid peroxidation decreased, at 24 and 48 hours post injection. However, upon bacterial challenge, regardless of the effect of seaweed extracts, a decreasing trend of hematocrit values was observed although there was no statistical difference among the fish groups. However, there is no beneficial effect of seaweed extracts on hematocrit upon bacterial infection since there were no differences observed in hematocrit values between fish injected with NaCl and seaweed extracts.

The complement, a major mediator of innate immune defense against infection via inflammation, opsonization and cell lysis, involved in killing pathogenic microorganisms (Mayilyan *et al.*, 2006). It plays an essential role in alerting the host immune system to the presence of the pathogens. The complement cascade is initiated by one or a combination of three pathways, namely, the classical, alternate, and lectin. Among them, the alternative complement pathway is of great importance in the innate immune response in teleost fish (Yano, 1996). This pathway is independent of antibody and activated directly by foreign microorganisms. Injection of sodium alginate extracted from brown algae (*M. pyrifera*) or iota-carrageenan from red algae (*Chondrus crispus*) at 20 and 30 mg kg⁻¹ was reported to increase the alternative complement activity against *V. alginolyticus* after 24 and 72 hours, and after 120 hours, slightly decreased to the original level (Cheng *et al.*, 2007). The critical period of *S. iniae* infection has been reported within 24 to 48 hours which caused a mass mortality

in the culture of Asian seabass (Taniguchi, 1983; Bromage *et al.*, 1999). In the present study, the alternative complement activities decreased at 24 hours post injection of *S. iniae*. This demonstrated that *Sargassum* sp. extract may enhance the stimulation of the alternative complement activity.

Lysozyme is considered as one of the important antibacterial molecules in fish. It disrupts bacterial cell walls by splitting glycosidic linkages in the peptidoglycan layers of bacteria (Ellis, 1999; Magandottir *et al.*, 2005; Wang *et al.*, 2010) after the complement and other enzymes have disrupted the outer walls (Yano *et al.*, 1996). Experiments with white shrimp showed that lysozyme activity was enhanced when *L. vannamei* were immersed in hot water extract from *S. hemiphyllum* var. *chinense* (Huynh *et al.*, 2011) and when *Fenneropenaeus chinensis* were fed with extract from *S. fusiforme* (Huang *et al.*, 2006). In addition, lysozyme activity of grouper, *Epinephelus coicoides*, intraperitoneally injected with sodium alginate from brown algae (*Macrocystis pyrifera*) and iota-carrageenan from red algae (*C. crispus*) were significantly higher than those of fish injected without sodium alginate and iota-carrageenan after 24 and 72 hours post challenge and slightly returned to the original level after 120 hours (Cheng *et al.*, 2007). In the present study, lysozyme activity of fish decreased at 24 hours and increased at 48 hours post injection especially in seaweed extract injected fish, indicating some effect of *Sargassum* sp. extract on the recovery of lysozyme. Upon bacterial injection, the lysozyme activity of fish which were injected with seaweed extracts was higher than those of fish which were not injected with the seaweed extract, although

it was not statistically different, except at 48 hours.

Lipid peroxidation is a well-defined mechanism of cellular damage in both animals and plants which is caused by several factors. Infection in fish is one of the reasons for the increase in lipid peroxidation (Mohankumar and Ramasamy, 2006). The level of malondialdehyde (MDA) has been used as an indicator for lipid peroxidation. Aydin *et al.* (2009) reported that lipid peroxidation content in tissues of infected rainbow trout was significantly higher than that in healthy animals. Brown algae such as *Laminaria japonica*, *S. siliquastrum* and *S. polycystum* could prevent the increase of lipid peroxide (Li *et al.*, 2002; Lim *et al.*, 2002; Balaji-Raghavendran *et al.*, 2005). The results of lipid peroxidation from this study showed that the injection with bacteria *S. iniae* contributed to an increase of MDA level in the liver, compared to those injected with NaCl. It indicated that *Sargassum* sp. extract could suppress lipid peroxidation during bacterial infection.

CONCLUSION

No antibacterial activities of hot-water extract from *Sargassum* sp. against fish pathogens were observed, however, the extract of *Sargassum* sp. showed immunostimulatory effects on non-specific immunity responses as evidenced by increased serum lysozyme and alternative complement activities in Asian seabass infected with *S. iniae*. Moreover, the *Sargassum* extract could suppress the lipid peroxidation in the fish liver caused by bacterial infection.

It could be considered that the hot-water extract of *Sargassum* sp. should be used as an immunostimulant by injection; however, continuous administration might be necessary to ensure the immunity of Asian seabass. Further research should be focused on the effect of *Sargassum* sp. hot-water extract supplementation in fish feed on the immune responses and resistance against *S. iniae* infection.

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